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Antigenicity of *Streptococcus agalactiae* extracellular products and vaccine efficacy

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Abstract

Streptococcus agalactiae is a major bacterial pathogen that is the cause of serious economic losses in many species of freshwater, marine and estuarine fish worldwide. A highly efficacious *S. agalactiae* vaccine was developed using extracellular products (ECP) and formalin-killed whole cells of *S. agalactiae*. The vaccine efficacy following storage of *S. agalactiae* ECP and formalin-killed *S. agalactiae* cells at 4 °C for 1 year was determined. The stored ECP containing *S. agalactiae* formalin-killed cells failed to prevent morbidity and mortality among the vaccinated fish, and the relative percentage survival was 29. Serum antibody responses of the stored ECP and freshly prepared ECP against soluble whole cell extract of *S. agalactiae* indicated that significantly less antibody was produced in fish immunized with stored ECP and *S. agalactiae* cells than in those fish immunized with freshly prepared ECP and *S. agalactiae* cells at day 31 post-vaccination. Silver staining of sodium dodecyl sulphate-polyacrylamide gels and immunostaining of Western blots with tilapia antiserum to *S. agalactiae* revealed that predominant 54 and 55 kDa bands were present in the freshly prepared ECP fraction. The 55 kDa band was absent from the stored ECP and new bands below 54 kDa appeared on the Western blot. The results of this study on *S. agalactiae* ECP provide evidence for a correlation between protection and antibody production to ECP and for the importance of the 55 kDa ECP antigen for vaccine efficacy.

Keywords: efficacy, extracellular products, Nile tilapia, serum antibody, *Streptococcus agalactiae*, vaccine.

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Introduction

Piscine group B streptococcal infections have been associated with significant morbidity and mortality among freshwater, estuarine and marine fish species. Pathogenesis in fish involves septicaemia and colonization of numerous organs, such as the nares, brain, kidney and intestines; clinical signs appear soon after infection, and include depression or excitability, anorexia, 'C'-shaped body posturing, erratic swimming and whirling, and death (Baya, Lupiani, Hetrick, Roberson, Lukacovic, May & Poukish 1990; Eldar, Bejerano, Livoff, Hurvitz & Bercovier 1995; Evans, Klesius, Glibert, Shoemaker, Al Sarawi, Landsberg, Duremdez, Al Marzouk & Al Zenki 2002). Significant bacterial susceptibility among group B streptococcal fish pathogens has been reported to a number of antibiotics, including oxytetracycline, penicillin, ciprofloxacin, chloramphenicol and rifampin (Robinson & Meyer 1966; Baya *et al.* 1990; Evans *et al.* 2002). However, no antibiotics are currently approved by the United States Food and Drug Administration for use against streptococcal infections in fish (Center for Veterinary Medicine 2003).

An emerging group B streptococcal fish pathogen, *Streptococcus agalactiae*, has been shown to affect numerous fish species, including tilapia,

Oreochromis niloticus (L.), and mullet, *Liza klunzingeri* (Day) (Evans *et al.* 2002), golden shiners, *Notemigonus crysoleucas* (Mitchill) (Robinson & Meyer 1966), menhaden, *Brevoortia patronus* Goode (Plumb, Schachte, Gaines, Peltier & Carroll 1974), and bullminnows, *Fundulus grandis* Baird & Girard (Rasheed & Plumb 1984). Outbreaks with considerable mortalities have been associated with multiple environmental factors, including warm water temperatures, increased ammonia levels and low dissolved oxygen levels (Evans *et al.* 2002). Several killed and modified-killed vaccines have been tested against piscine streptococcal disease caused by *S. difficile* (Eldar, Shapiro, Bejerano & Bercovier 1995), *Enterococcus* sp. (Romalde, Silva, Riaza & Toranzo 1996), *Streptococcus* sp. (Akhlaghi, Munday & Whittington 1996) and *S. iniae* (Eldar, Horovitcz & Bercovier 1997; Klesius, Shoemaker & Evans 2000). Evans, Klesius & Shoemaker (2004a), Evans, Klesius, Shoemaker & Fitzpatrick (2004b) and Evans, Shoemaker & Klesius (2004c) evaluated the efficacy of a killed vaccine composed of concentrated extracellular products (ECP) and formalin-killed *S. agalactiae* whole cells.

Determination of vaccine stability and potency is vital to satisfy practical product considerations and government regulatory issues. Such characterization is important here, because previous studies have indicated that the efficacy of mammalian group B streptococcal capsular polysaccharide-protein conjugate vaccines can significantly decrease when stored for 3 years in aqueous form at 2–8 °C (Paoletti 2001). Fish farmers may wish to vaccinate their fish with the piscine *S. agalactiae* vaccine at any time during a production cycle or during multiple production cycles, and a vaccine with a long shelf life would therefore be desirable.

In the present study, the goal was to determine the efficacy of the *S. agalactiae* vaccine after storage and to characterize the immunogenic components of *S. agalactiae* ECP and their stability in freshly prepared *S. agalactiae* vaccine compared with those in 1-year stored *S. agalactiae* vaccine. The specific objectives were to: (i) assess the efficacy of the *S. agalactiae* vaccine stored for 1 year at 4 °C, (ii) evaluate the production of ECP-specific antibodies and their association with vaccine efficacy following immunization with the freshly prepared or stored vaccine, and (iii) identify ECP antigens from the vaccine recognized by Western blot developed with serum antibodies from tilapia surviving *S. agalactiae* infection.

Materials and methods

Fish

Nile tilapia were obtained from stocks maintained at the USDA/ARS Aquatic Animal Health Research Laboratory in Auburn, AL, USA. The fish had a mean weight of 30.3 ± 1.95 g and were housed at the USDA/ARS AAHRL in Chestertown, MD, USA, for the study. The fish were kept in 57-L glass aquaria supplied with flow-through dechlorinated tap water and were maintained on a 12 h:12 h light:dark period. The fish were fed daily to satiation with Aquamax Grower 400 (Brentwood, MO, USA).[1] Daily water temperature averaged 30.87 ± 1.37 °C, mean daily dissolved oxygen was 4.26 ± 0.90 mg L^{-1}, and mean ammonia concentration was 1.05 ± 0.61 mg L^{-1}.

Vaccination

The *S. agalactiae* vaccine was prepared as previously described and standardized according to determined pH, salinity, refractometer, and optical density values (Evans *et al.* 2004a; Evans *et al.* 2004c). The polysaccharide-encapsulated *S. agalactiae* was grown in tryptic soy broth (TSB, Difco Laboratories, Sparks, MD, USA) and incubated at 27 °C for 72–125 h. The resulting cultures were treated with 3% neutral buffered formalin for 24 h and then centrifuged to separate the cell pellet and culture fluid. The ECP fraction of the vaccine was prepared by concentrating the cell-free culture fluid containing ECP on a 3-kDa Amicon column (S3Y3) using a Millipore Proflux M12 (Millipore, Billerica, MA, USA), and sterilized using a 0.22 μm 1-L microbiological filter (Corning, Corning, NY, USA). The concentrated ECP fraction and the whole cell fractions were stored separately in sterile containers at 4 °C for 1 year until use in the vaccination study. In addition, fresh encapsulated *S. agalactiae* vaccine was prepared just prior to the beginning of the study to inoculate fish for blood samples.

On the day that fish were injected (day 0 post-vaccination), 16 mL of the formalin-killed cells were added to 1 L of the sterilized, concentrated cell-free culture fluid with concentrated ECP. The concentration of this preparation was approximately

[1]Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

4×10^9 colony-forming units (CFU) per mL of *S. agalactiae*. Vaccine sterility was determined by streaking a vaccine sample on sheep blood agar (SBA; Remel, Lenexa, KS, USA) at 35 °C for 72 h. Triplicate groups of 15 fish each were injected intraperitoneally (i.p.) with 0.1 mL of the 1-year-old stored *S. agalactiae* vaccine (on day 0 post-vaccination), while additional triplicate groups of 15 fish each were injected i.p. with 0.1 mL sterile TSB to serve as controls. After injection, all fish were kept in aquaria and maintained as previously described.

Experimental challenge

On day 31 post-vaccination (corresponding to day 0 post-challenge), fish from the 1-year-old stored vaccine and TSB control groups were challenged i.p. with 1.7×10^4 CFU *S. agalactiae* per fish. Fish were monitored daily for clinical signs of disease and mortality for 21 days post-challenge. Moribund and dead fish were removed twice daily, and bacterial samples were aseptically obtained from the nares, brain, head kidney, and intestine of 20% of morbid and dead fish to confirm the presence of *S. agalactiae*. Samples were cultured at 35 °C for 24 h on SBA, and isolate identity was confirmed as *S. agalactiae* using the BIOLOG MicroLog Microbial Identification System (BIOLOG, Hayward, CA, USA) according to the manufacturer's instructions. Positive cultures exhibited beta-haemolytic, oxidase-negative, catalase-negative, Gram-positive cocci (Evans *et al.* 2002). Over the 21-day period of observation, the mean percentage mortality and mean percentage cumulative mortality of treatment and control fish were determined. The efficacy of the vaccine was assessed as the relative percentage survival (RPS) (Amend 1981).

Enzyme-linked immunosorbent assay

Two studies were conducted based on the indirect enzyme-linked immunosorbent assay (ELISA) methods of Shelby, Klesius, Shoemaker & Evans (2002). For the first ELISA, tilapia inoculated with TSB, stored *S. agalactiae*, or fresh *S. agalactiae* vaccine (using the methods described in *Vaccination*) were bled from the caudal vein on days 0 and 31 post-vaccination. The blood samples were held at 25 °C for 1 h, and serum was separated with centrifugation at 400 ***g*** for 6 min and then stored at −70 °C until use. The ELISA antigen was prepared by sonication of whole encapsulated *S. agalactiae* cells followed by centrifugation at 4000 ***g*** for 20 min. The resulting supernatant was removed, and total protein content of this fraction determined by the bicinchoninic acid method and then adjusted to 500 μg protein mL^{-1}. For the second ELISA, immune serum was obtained from tilapia injected with whole *S. agalactiae* cells. The fish were bled from the caudal vein 30 days later, the blood samples were held at 25 °C for 1 h, and serum was separated by centrifugation at 400 ***g*** for 6 min and then stored at −70 °C until use. The ELISA antigen was prepared by concentration of the stored or fresh ECP fraction as described in *Vaccination*. The total protein content of this solution was determined by the bicinchoninic acid method and then adjusted to 500 μg protein mL^{-1}.

After the initial steps, all the assays were completed using the following steps. A quantity of 100 μL of antigen was added to each well of a 96-well microtitre plate, which was incubated at 25 °C for 2 h. The wells were blocked with 3% bovine serum albumin (Sigma, St Louis, MO, USA) at 25 °C for 1 h, and then the plates were washed with phosphate-buffered saline plus 0.05% Tween-20 (PBS-T). Nile tilapia serum samples were diluted 1:100 in PBS-T and 100 μL of the resulting solution was added to three replicate wells of the microtitre plate. The plate was incubated at 25 °C for 1 h and then washed with PBS-T. Mouse anti-tilapia IgM heavy chain specific monoclonal antibody 1H1 (Shelby *et al.* 2002) was diluted 1:5000 in PBS-T and 100 μL of this solution added to each well. The plate was incubated at 25 °C for 1 h and washed again with PBS-T. Peroxidase-conjugated rabbit anti-mouse IgG (Pierce Biotechnology, Rockford, IL, USA) was diluted 1:5000 in PBS-T and 100 μL of this solution was added to each well. The plate was washed again and 100 μL of One-Step Ultra TMB-ELISA (Pierce) was added to each well. The ELISA reaction was stopped at 20 min with 50 μL 3 M H_2SO_4, and the optical density of the reactions were read at 450 nm with a Bio-Tek Automated Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA).

SDS-PAGE and Western blot

Concentrated stored and freshly prepared ECP fractions were used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

and Western blotting techniques to compare their immunoreactive ECP antigens against immune serum from *S. agalactiae*-exposed tilapia. A quantity of 50 mL of culture supernatant was concentrated 10-fold to a final volume of 5 mL using microcentrifuge filters (Ultrafree CL Filter Units, 10 000 NMWL, Sigma).

The SDS-PAGE protocol was performed based on the method of Laemmli (1970) and used Criterion 10–20% precast slab gels (Bio-Rad, Hercules, CA, USA). The *S. agalactiae* ECP fraction was mixed 2:1 with SDS-PAGE sample buffer without 2-β-mercaptoethanol and samples were separated by electrophoresis at 175 V in a Mini electrophoresis cell (Bio-Rad). Following electrophoresis, the gel was stained with silver nitrate using the GelCode-SilverSNAP staining kit (Pierce) according to the instructions provided.

Unstained gel containing the resolved *S. agalactiae* ECP fraction was electroblotted onto a nitrocellulose membrane according to the method of Towbin, Staehelin & Gordon (1979) using a Criterion electroblotting unit (Bio-Rad). Following transfer at 100 V for 30 min, the nitrocellulose membrane was equilibrated for 30 min in StartingBlock blocking buffer (Pierce). The blot was rinsed and incubated in antiserum from *S. agalactiae*-exposed tilapia. The fish were bled from the caudal vein 30 days after exposure, the blood samples were held at 25 °C for 1 h, and serum was separated with centrifugation at 400 ***g*** for 6 min and then stored at −70 °C until use. For the Western blot, the serum was diluted 1:50 in 0.2 M Tris-buffered saline (TBS) and applied to the blot. After 1 h incubation, the blot was then rinsed and treated with mouse anti-tilapia IgM heavy chain-specific monoclonal antibody 1H1 diluted 1:5 in TBS. Alkaline phosphatase-conjugated anti-mouse immunoglobulin (Jackson ImmnoResearch, West Grove, PA, USA) at a dilution of 1:5000 was used for detection-specific antibody-bound antigen bands following treatment with the 1-Step NBT/BCIP colour-developing reagent (Pierce).

Statistics

Mortality data between vaccinated and control groups was analysed by *t*-test using the SAS program (SAS Institute, Cary, NC, USA). Mortality data and ELISA results were compared using one-way analysis of variance and Duncan's multiple range test. Significant differences between groups were accepted at $P < 0.05$.

Results

Fish were challenged with live *S. agalactiae* on day 31 post-vaccination (day 0 post-challenge) and subsequent mortality patterns were monitored. Control and vaccinated fish began to display clinical signs of disease 24 h after challenge, including lethargy, anorexia, changes in colouration and erratic swimming. Many of the fish also had long mucoid faecal casts trailing from the anus. The majority of mortalities in both groups occurred during the first 5 days post-challenge (Fig. 1). Over the course of 21 days post-challenge, the control group experienced 45% mortalities while the group vaccinated with stored vaccine experienced 32%. Given that tilapia surviving until the end of the study lived for 21 days, the mean survival time was 14.7 ± 8.2 days for the controls and 16.4 ± 7.5 days for the vaccinates. The mortality patterns failed to exhibit any significant differences between the groups and the RPS at day 21 post-challenge was 29.

Samples for microbiological examination were taken from 10 fish from each group and cultured on SBA at 35 °C for 24 h. All fish from each group were culture positive for *S. agalactiae*. All samples from the brain and head kidney were positive for *S. agalactiae* growth, while 70% of the nares samples and 60% of the intestine samples were positive. The lowest percentage of culture-positive organs was found in the intestine (40%) and nares (60%) samples from the group administered the 1-year-old stored vaccine.

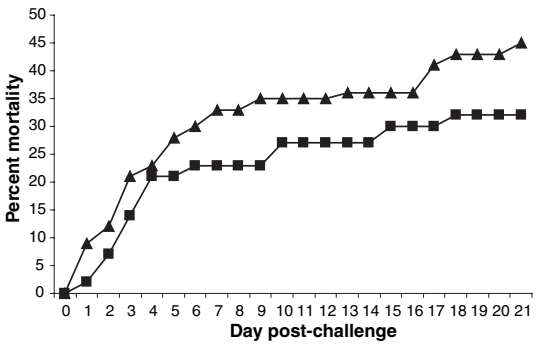

Figure 1 Post-challenge mortality patterns among tilapia injected with TSB as control (▲) or stored *Streptococcus agalactiae* vaccine (■) and then challenged i.p. with 1.7×10^4 CFU *S. agalactiae* per fish 31 days later.

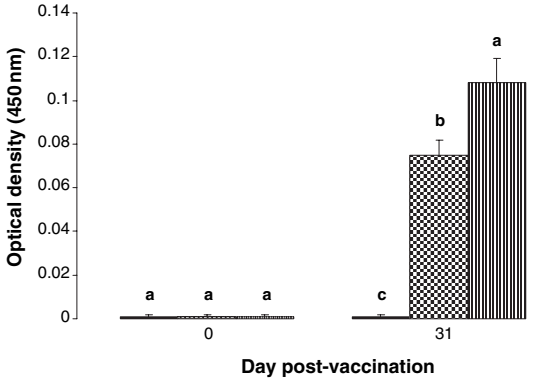

Figure 2 Production of anti-*Streptococcus agalactiae* antibodies (ELISA optical density) in tilapia injected i.p. with TSB as control (solid black bars), stored *S. agalactiae* vaccine (chequered bars), or fresh *S. agalactiae* vaccine (vertical striped bars). Serum samples were taken from fish on days 0 and 31 post-vaccination and *S. agalactiae*-specific antibody production was evaluated by ELISA. Significant differences between groups within each sampling day are noted by different letters.

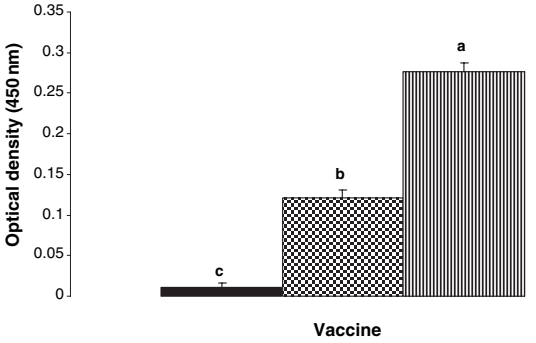

Figure 3 ELISA results (optical density) for wells coated with antigen from carbonate buffer as control (solid black bar), extracellular products (ECP) from stored *Streptococcus agalactiae* vaccine (chequered bar), or ECP from freshly prepared *S. agalactiae* vaccine (vertical striped bar). Wells were incubated with serum from fish immunized with *S. agalactiae*. Significant differences between groups within each sampling day are noted by different letters.

The results from the first ELISA experiment showed that no specific antibodies existed in any fish on day 0 before vaccination. On day 31 post-vaccination, the TSB controls also did not exhibit antibody production after injection, but the fish administered the stored vaccine or the fresh vaccine showed significant increases in *S. agalactiae*-specific antibody production (Fig. 2). These increases were approximately 75- (stored vaccine) and 108-fold (fresh vaccine) higher than the levels of all the groups from day 0 and the TSB control group from day 31 post-vaccination. In addition, the fish administered the fresh vaccine had significantly higher levels of serum antibodies on day 31 post-vaccination than the fish administered the stored vaccine. The latter group exhibited 69% of the antibody level of the fish given the fresh vaccine.

In the second ELISA experiment, freshly prepared or stored vaccine ECP was used as the target antigen and incubated with the positive fish serum. The OD for the wells coated with stored vaccine ECP was significantly lower (2.2-fold) than the OD for the wells coated with freshly prepared ECP (Fig. 3). The ECP from both freshly prepared and stored solutions produced significantly higher OD values than wells coated with coating buffer only.

Freshly prepared ECP and stored ECP fractions were silver stained on SDS-PAGE and immunostained by Western blot with serum from *S. agalactiae*-exposed tilapia. Silver staining of the SDS-PAGE indicated banding differences between the ECP fractions from the two different samples (Fig. 4). The freshly prepared ECP fraction sample exhibited bands at approximately 47 and 75 kDa with predominant bands at 54 and 55 kDa. The stored ECP fraction showed weakly stained bands at approximately 47, 54 and 55 kDa, and no banding at 75 kDa. The 54 and 55 kDa bands showed reactions in Western blots of the freshly prepared ECP fraction, while the 55 kDa band was absent and new, weak bands below 54 kDa appeared with the stored ECP fraction.

Discussion

In our previous studies, inoculation with fresh vaccine significantly prevented development of clinical signs and reduced the post-challenge mortalities caused by *S. agalactiae*. Consistent protection was conferred regardless of vaccine lot, as vaccine construction was standardized and serial preparations had the same immunogenic activity. Previously, intraperitoneal injection of tilapia with fresh polysaccharide-encapsulated *S. agalactiae* vaccine uniformly yielded an RPS of approximately 80 after bacterial challenge at 30–32 °C (Evans *et al.* 2004b,c). However, the stored vaccine studied here failed to significantly protect immunocompetent tilapia originating from the same source of fish as used in our previous studies. The TSB control fish experienced decreased mean days of survival and increased percentage mortalities compared with fish

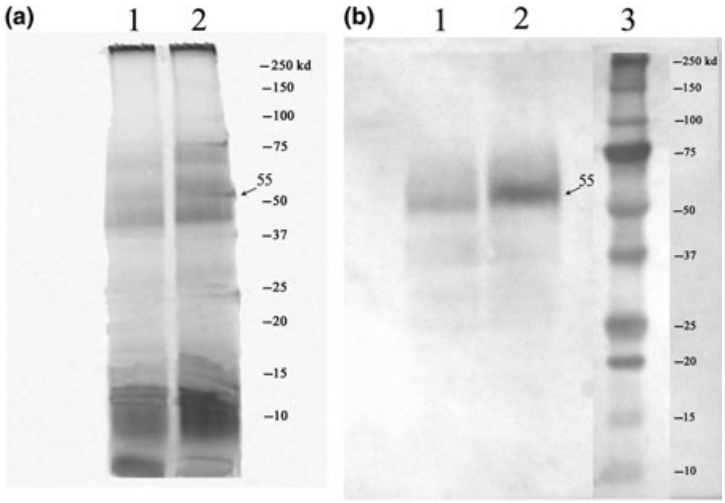

Figure 4 SDS-PAGE banding and Western blot reactivity of extracellular products (ECP) from stored and freshly prepared *Streptococcus agalactiae* vaccines. (a) SDS-PAGE-resolved, silver-stained *S. agalactiae* ECP fraction; lane 1, following storage at 4 °C and lane 2, freshly prepared ECP fraction. Predominant 54 and 55 kDa bands are more prominent in lane 2. (b) Identical samples resolved on one half of the same gel, blotted onto nitrocellulose membrane and probed with pooled immune serum from *S. agalactiae*-exposed tilapia and mouse anti-tilapia IgM heavy chain-specific monoclonal antibody 1H1 as second antibody. An antigen with an apparent molecular mass of 55 kDa is indicated by the arrow. Lane 3, Recombinant Precision Plus (Bio-Rad) prestained molecular weight standard.

injected with the 1-year-old stored vaccine. Although this raw mortality data suggested that injection with the stored vaccine enhanced survival after bacterial challenge, statistical analysis found no significant differences between the mortality patterns of the control and vaccinated groups. Furthermore, injection with the stored vaccine allowed development of clinical signs and only resulted in an RPS of 29. While the first ELISA study indicated that the 1-year-old stored vaccine induced significantly increased antibody production in vaccinates compared with the TSB controls, this level of antibody production was significantly lower than found in our studies using fish injected with fresh *S. agalactiae* vaccine. Because immunity against streptococcal infection is dependent on specific antibody responses (Klesius, Zimmerman, Mathews & Krushak 1974; Shelby *et al.* 2002), the decreased antibody levels in fish administered stored vaccine presumably account for the decreased RPS.

Extracellular products are important virulence factors of fish pathogens and are often sufficiently immunogenic to provide protection against challenge after inoculation. In fact, ECP antigens have been used to construct a number of efficacious vaccines against piscine pathogens such as *Photobacterium damselae* spp. *piscicida* (Magariños, Romalde, Santos, Casal, Barja & Toranzo 1994), *Vibrio harveyi* (Zorrilla, Arijo, Chabrillon, Diaz, Martinez-Manzanares, Balebona & Moriñigo 2003) and *Flavobacterium psychrophilum* (LaFrentz, LaPatra, Jones & Cain 2004). The efficacy of these vaccines was often enhanced by selecting and concentrating certain ECP antigens shown to be highly immunogenic and protective.

In this study, the production of specific anti-ECP antibodies was associated with efficacy of the *S. agalactiae* vaccine. The level of protection in vaccinated fish against *S. agalactiae* challenge corresponded to the level of antibodies produced against the ECP. This conclusion was illustrated by the second ELISA, which showed that serum from *S. agalactiae*-exposed tilapia had a 44% decrease in recognition of stored ECP compared with the freshly prepared ECP. This change in vaccine immunogenicity and protection after storage was reflected by the SDS-PAGE silver staining and Western blot immunostaining, which showed a change in the ECP profile between the freshly prepared and stored vaccine samples. The freshly prepared ECP fraction sample exhibited bands at 47 and 75 kDa and predominant bands were observed at 54 and 55 kDa. Because the 47, 54 and 55 kDa bands were lighter and the 75 kDa band was absent in the stored vaccine sample, some antigenic loss apparently occurred during vaccine storage. Western blots immunostained using serum from *S. agalactiae*-exposed tilapia showed strong

reactions with the 54 and 55 kDa antigens of the freshly prepared ECP fraction. Nonetheless, the serum did not react with the 47 and 75 kDa ECP fraction components visualized by SDS-PAGE silver stain, indicating that these components are not important immunostimulatory components of the vaccine. This suggests that the 54 and 55 kDa antigens are the immunodominant antigens in the vaccine. However, the stored ECP SDS-PAGE and Western blot lanes only showed absent or altered reactions, indicating that the immunodominant antigens are quantitatively reduced in the stored vaccine. Further, the new, weak bands below 54 kDa band from the Western blot suggest that these antigens degraded to slightly lower molecular weights during storage. The apparent loss of ECP antigen and antigen recognition may account for the significantly decreased immune responses and survival patterns among fish injected with stored vaccine.

The definitive mechanism for the antigen loss was not elucidated here, but a similar phenomenon was observed in another study and suggests the cause of this loss. Paoletti (2001) assessed the potency of multiple-conjugated group B streptococcal vaccines and found that some of the vaccines lost over 50% potency when stored for 3 years in aqueous form at 2–8 °C. The author proposed that the ketosidic bond between the galactose and sialic acid components of the streptococcal organisms might have been cleaved by acid hydrolysis. Previous studies have shown that sialic acid is immunologically important because it controls the structural conformation of immunodominant epitopes (Jennings 1992; Baker 1993). If changes in the antigenic structure occurred due to bond cleavage, then the antigens of *S. agalactiae* would fail to provide sufficient immunostimulation. Some antigens may still be available in sufficient concentrations to stimulate specific antibodies that bind the ELISA whole cell antigen preparation, but the overall antibody response might decrease below a protective level.

Only a limited number of studies have assessed the potential of long-term storage of aquatic animal vaccines. However, these studies indicated that proper vaccine storage could be employed without diminishing immunogenic and protective effects. Marsden, Vaughan, Fitzpatrick, Foster & Secombes (1998) found that a freeze-dried live preparation of an *Aeromonas salmonicida* vaccine was able to stimulate the same level of antibody production and lymphocyte proliferation as fresh-grown bacteria. The authors proposed that the shelf life of the freeze-dried preparation was at least 12 months. Alabi, Jones & Latchford (1999) assessed a freeze-dried preparation of a killed *Vibrio harveyi* vaccine and suggested that it provided penaeid larvae with an equal degree of protection against challenge as did a fresh vaccine preparation. Wise, Klesius, Shoemaker & Wolters (2000) immersed fish in a freeze-dried, live, attenuated *Edwardsiella ictaluri* vaccine and found significantly lower mortalities after bacterial challenge among vaccinated fish than unvaccinated fish. Although these authors did not predict the potential long-term stability of the vaccines, freeze-dried preparations presumably have considerably longer shelf lives. Therefore, the effects of other commonly used vaccine preparation techniques such as freeze-drying should be assessed to help extend the shelf life of the vaccine. This study suggests that the shelf life of the *S. agalactiae* vaccine is < 1 year when stored at 4 °C in its aqueous form, indicating that a fresh preparation of the vaccine should be utilized to obtain its full benefits.

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